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Natural products, deforestation, and wildlife tourism could be a potential threat due to leptospirosis outbreak in Gashaka Gumti national park, and the *in vitro* antileptospiral activity of *Annona senegalensis*, its synergistic effects with commonly prescribed antibiotics attempts

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Abstract

Introduction: The research on biodiversity as regards natural products, deforestation, and wildlife tourism has witnessed a tremendous growth as several scholars from different jurisdictions have directed their attention toward this subject matter. This growth shows the importance to academia in addressing the critical issues of health of biodiversity conservation. Leptospirosis is a worldwide infectious and zoonotic disease. The incidence of this disease is high in temperate regions, the plant *Annona senegalensis*, also known as wild custard apple and wild soursop is a member of Annonaceae family. It is a fruit tree native to Senegal and found in semi-arid to sub humid regions of Gashaka Gumti National Park, with a long history of traditional use. Numerous ethnomedicinal uses have been attributed to different parts of *A. senegalensis*, as well as its use as food and food additives. The aim of this study was to evaluate the natural products, deforestation, and wildlife tourism a potential threat due to Leptospirosis outbreak in Gashaka Gumti National Park and investigate the effects of plant extract found in Gashaka gumti national park plant extract on pathogenic antileptospiral properties of Annona senegalensis against pathogenic Leptospira species (spp.) and to study its synergistic effects with commonly prescribed antibiotics.

Materials and Methods: The tested *Leptospira serovars* were *Australis, Bataviae, Canicola* and *Javanica*. Solvent extract of hexane, dichloromethane, chloroform, ethyl acetate and methanol extracts were used. Broth dilution methods were used to determine the Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC) and the synergistic effects between the Solvent extract and the tested antibiotics. The synergistic effects was evaluated by using the fractional inhibitory concentration (FIC) index. Morphological changes of the treated *Leptospira* were observed under a Scanning Electron Microscope (SEM).

Results: The ASH, ASD, ASC, ASEt, ASM and ASEtol were found to have antileptospiral properties against the tested *Leptospira* spp. The synergy result showed that only combination of ASM ASEt and ASEtol and penicillin G against serovar Australis has demonstrated synergistic effect with the FIC index of 0.38. Morphological study using SEM showed significant structural changes of the treated *Leptospira* spp.

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Conclusions: The result suggests that Annona senegalensis extracts could potentially be used as either a complimentary or an alternative therapeutic agent against pathogenic *Leptospira* spp which will help in the curtailing natural products, deforestation, and wildlife tourism threat due to Leptospirosis.

Keywords: Natural products; Deforestation; Wildlife; Tourism; Threat; Leptospirosis; Gashaka Gumti; National Park; Antibiotics

1. Introduction

The research on biodiversity as regards natural products, deforestation, and wildlife tourism has witnessed a tremendous growth as several scholars from different jurisdictions have directed their attention toward this subject matter [1]. This growth shows the importance to academia in addressing critical issues of health of biodiversity conservation society is grappling with over the last decade.

The increasing attention on biodiversity conservation is due to fact its intended value is being threatened at a faster rate [1] which has led to a considerable loss of wild life and their habitat relative to biological average rate [2].

The fast decline of endanger species and habitat has been attributed to imprints of pathogens, which started many years ago, when infections like *leptospiral* bacterial effects on plants, animals and human to some extent. The threat to diversity is not limited only loss of endangered species, but loss of habitat due to this bacterial inversion, with the expected human growth of 8.3 billion and average life expectancy exceeding 85 years in 2030 globally, demands of societies and households will be more diverse than anticipated. The consequence there of will further enlarge the imprints of pathogen leading to rapid transformation of habitat into communities and increasing economic activities thereby destroying the entire biotic resources [3]

In response to the present rate of extinction of species as a result of an infections from Leptospirosis, conserving biodiversity is warranted to stem the tide, safeguard and protect the existing species [4]. This initiatives to evaluate the activity of plant against Leptospirosis have been taken to harness efforts to curtail further loss of biodiversity from this pathogens

Leptospirosis is a contagious zoonotic disease spread worldwide and Leptospirosis is caused by a bacterium called *Leptospira interrogans*. The organism is carried by many animals and lives in their kidneys. It ends up in soil and water through their urine. The genus *Leptospira i*ncludes pathogenic, intermediate, and non-pathogenic species with the pathogenic species causing infection in humans and animals [5]. *Leptospirosis* is widespread throughout the world and it is endemic in tropical regions with high rainfall [6]. The World Health Organization recognizes leptospirosis, also known as Weil's disease, as a neglected tropical disease with a significant global health burden. Globally, it has been estimated to be 1.03 million cases annually with 58,900 deaths [7].

The *Leptospira* are commonly found in rodents, as well as variety of wild and domestic animals, livestock, and insectivores [8]. Infection may occur through direct contact with an infected animal as well as through indirect contact via soil or water contaminated with urine from an infected animal. People as risk are those that are in direct contact with potentially infected animals, such as the veterinarians, farmworkers, hunters, animal shelter workers, scientists, and technologists handling animals in laboratories. The family *Leptospiraceae* contains two genera, *Leptospira* and *Leptonema*. Based on antigenic determinants, the genus *Leptspira* is classified into two species, *Leptospira interrogans* and *Leptospira biflexa,* the parasitic and saprophytic forms, respectively.

An effective course of treating *leptospirosis* still remains unsolved problem. *Leptospirosis* usually response to treatment with the antibiotics, provided they are administered in enough doses early in the infection. Benzyl penicillin should be administered intravenously for up to 7 days in a daily dose of 6-8 mega units (3.6-4.8 g) but penicillin may cause a temporary exacerbation of the symptoms. Tetracyclin should be administered if there is evidence of renal failure. Continuous renal replacement therapy is supposed to be superior to conventional haemodialysis in leptospirosis [9].

Gashaka Gumti national park is a wild world of wonders. The park is the largest, most biological diverse and scenic protected area in Nigeria. The main attraction for visitation to Gashaka Gumti park are its astonishing and inspiring wild life, its variety of fauna and flora, many swiftly flowering rivers, stiking mountains, deep valleys and geothermal springs that evolved over millions years ago. The park is a reputable open air Museum of natural History and material culture of the indigenous people as well as some colonial legacies of the German, British and French [9].

Figure 1 Gate way to Gashaka Gumti National park and the Park Rangers

The park has a unique habitats created by the presence of intricate vegetation –types mostly of medicinal values, a diverse range of topography, altitude that range from 450 to 2419m above the sea level north to south with climate that support the ecosystem of exceptionally high biodiversity. The park possesses important reserves of various vegetation-type anging from deciduous savannah woodland, montane rain forest and montane grasslands to relic moist –lowland rainforests. The park is the only protected area in West Africa that has such distinct ecological transition.

Figure 2 Montane Ecosystem of GGNP

The *Annona senegalensis* numerous species, some of them Afro-montane endemics, of which are threatened by unsustainable harvesting. Annona senegalensis, species presumed to possess certain medicinal properties for the cure of diseases and ailment.

Gashaka Gumti serves as a wildlife corridor in the north-easterly direction to Faro, Benoue and Boubanjida National Park in Cameroon. Together, these Parks harbour many of the big games formerly found throughout West Africa sub region. The sheer variety of different habitats within the park makes the area so uniquely rich in wildlife. Rain forest provides sanctuary for animals such as the Giant foreshog, Hybchoerusmeinertzhagen, Leopard, Panthera pardus, yellow-backed Duiker, Cephalopus silvicultor, Golden cat, Felis aurata, and different primate's species, woodland Savannah is home to the African Buffalo, Syncerus caffer, Lion, Elephant, and Wild dog, Lycaon pictus. This is in addition to various ungulates such as waterbuck, Kobus ellipsiprymnus, Roan antelope, Hippotragus equinus, and Giant eland.Tauratragus derbianus. Others include Kob and Hertbeest, Alcelapus buselaphus as well as the white Rhinoscerus.

Few among the animals of interest as to the effects of *Leptospirosis* outbreak in the park to be considered of value to be protected as shown below;

Figure 3 Chimpanzee and Baboon as the charismatic fauna of the scientific studies

Figure 4 Other animals in the park at treat of Leptospira (i –xiii)

Annona senegalensis, a wild custard apple locally known as Gwadan daji is a Small tree 2-6m tall but may reach 11 m under favourable conditions. The bark is smooth to roughish, silver grey or grey-brown. Leaves are alternate, simple,

oblong, ovate or elliptic, green to bluish green, almost without hairs on upper surface but often with brownish hairs on the lower surface. Flowers are up to 3 cm in diameter on stalks 2 cm long, solitary or in groups of 2-4, arising above the leaf axils. The fruits are formed from many fused carpels, fleshy, lumpy, egg shaped, 2.5-5 by 2.5-4 cm, ovoid or globose, unripe fruit green, turning yellow to orange on ripening (Fig. 5). Wild fruit trees of this species are found in semi-arid to sub-humid regions of Africa. The species occur along river banks, fallow land, swamp, forests and at the coast. It commonly grows as a single plant in the understorey of savannah woodlands. It is found growing throughout Gashaka Gumti National Park is a plant widely used for the treatment of human diseases and ailment. This study aims to investigate the in vitro anti-leptospiral activity of *Annona senegalensis* extracts alone and combined with Benzathine Benzylpenicillin, Amoxicillin, Azithromycin, Ciftraxone, Tetracycline and Doxycycline.

Figure 5 *Annona senegalensis* showing the Leaf, Fruits and Stem bark

2. Material and methods

2.1 Materials

Methanol (Merck, Darmstadt, Germany), Soxhlet apparatus (Electro thermal, Staffordshire, UK), vacuum rotary evaporator (Buchi, Flawil, Switzerland), freeze-dryer (Ilshin Lab. Co., Ltd., Gyeonggi-do, Korea), magnetic stirrer (Thermo Fisher Scientific, Shanghai, China), well plates (Nest Biotech Co., Ltd., Wuxi, Jiangsu, China, phosphate-buffered saline (PBS; Sigma-Aldrich, St. Louis, MO, USA, Broth Ellinghausen McCullough Johnson Harris (EMJH), DifcoTM Leptospira Medium Base EMJH (Becton, Dickinson and Company, Sparks, MD, USA), autoclaved (Tomy Kogyo Co., Ltd., Tokyo, Japan), 15-mL centrifuge tubes (Nest Biotech Co., Ltd., Wuxi, Jiangsu, China). EMJH Agar and BactoTM Agar (Becton, Dickinson and Company, Sparks, Maryland, USA), 0.22-µm syringe filter; Bioflow, Kuala Lumpur, Malaysia, Centrifuge tubes (Nest Biotech Co., Ltd., Wuxi, Jiangsu, China), Penicillin and ceftriaxone (F. Hoffmann-La Roche Ltd., Basel, Switzerland), doxycycline (Sigma-Aldrich, St. Louis, MO, USA), shaker (Jeio Tech, Daejeon, Korea), ultraviolet-visible spectrophotometer (Shimadzu, Kyoto, Japan), automatic enzyme-linked immunosorbent assay (ELISA) tray reader (Thermo Lab systems, Helsinki, Finland), microscope (Olympus, Tokyo, Japan), McDowell-Trumps fixative (Electron Microscopy Sciences, Hatfield, PA, USA), 0.1 M PBS, 1% osmium tetroxide, 0.1 M PBS, 100% acetone, 100% hexamethyldisilazane, (Electron Microscopy Sciences, Hatfield, PA, USA), SEM instrument (FEI, Brno, Czech Republic).

2.2 Sampling and Plant Extraction

Mature whole *Annona senegalensis* plants were harvested from Gashaka Gumti National Park, Gashaka Local Government, Taraba State Nigeria and sent to the Forest Research Institute Federal University Wukari for identification as FUW/FRI/156-3. (Figure 1). The leaves of the plants were then washed and dried in the shade, powdered, and stored in dry place at room temperature. The solvent extracts [Hexane (ASH), Dichloromethane (ASD), Chloroform (ASC) Ethyl acetate (ASEt), Methanol (ASM) and Ethanol (ASEtol)]. The extracts were prepared by adding 200 g of plant powder to 500 mL of each solvents separately and soaking for 72 hrs with continuous stirring using a magnetic stirrer (500 rpm) at room temperature followed by evaporation to dryness using a vacuum rotary evaporator (337 mbar) at 250 rpm and 40 ^{o}C [11, 12]. Concentrated filtered solutions were freeze-dried into a powder and stored. Extract stock solutions were prepared by dissolving 8 mg of plant powder in 1 mL of phosphate-buffered saline. A filtered working solution (3200 µg/mL) was used immediately after preparation.

2.3 Bellingshausen McCullough Johnson Harris Broth Preparation

Bellingshausen McCullough Johnson Harris (EMJH) broth was prepared by dissolving DifcoTM Leptospira Medium Base EMJH in distilled water (2.3 g/L), which was autoclaved and left to cool. DifcoTM Leptospira Enrichment EMJH (100 mL/L) and filtered with 0.22-µm syringe filter, and 5-fluorouracil (200 µg/mL) was added. The broth was transferred to 15-mL centrifuge tubes. EMJH agar was prepared by dissolving DifcoTM Leptospira Medium Base EMJH (2.3 g/L) and BactoTM Agar (9 g/mL) in distilled water, followed by autoclaving, leaving to cool, and then adding DifcoTM Leptospira Enrichment EMJH (100 mL/L) supplemented with filtered 5-FU (200 µg/mL). The solution was poured into plastic petri dishes. Both EMJH broth and agar were incubated at 30–35 °C for 24 h for sterility testing and stored in a cold room prior to use.

2.4 Antibiotic Solution Preparation

Penicillin G, ceftriaxone, Amoxycline and doxycycline stock solutions were prepared by dissolving 1 g of powder to 1 mL of sterile distilled water. The working solutions (25 µg/mL) were used immediately after preparation. Leptospira Inoculum Preparation Reference strains of the Leptospira interrogans serovars Australis, Bataviae, Canicola were obtained from Universiti Malaysia Sarawak, and L. interrogans serovar Javanica cultures were obtained from the Institute for Medical Research Universiti Malaysia Sarawak, Malaysia. The strains were sub cultured and incubated with shaking at 30 °C and 40 rpm for seven days. The resultant cultures were diluted in EMJH broth to an optical density of 0.32 at 420 nm (approximately 1 × 108 colony-forming unit per millilitre (CFU/mL)) by an indirect method using an ultraviolet-visible spectrophotometer and then further serial-diluted to 2 × 106 CFU/mL in EMJH broth [13].

2.5 Antimicrobial Susceptibility Testing

The broth micro dilution method was used to determine the susceptibility of *Leptospira* species to penicillin, ceftriaxone, Amoxycline and doxycycline. The results were interpreted based on minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) [14]. One hundred microliters of EMJH medium broth was transferred into wells in 96-well plates, except for the first column. One hundred microliters of working extract or antibiotic solutions was added to the first column with a final volume of 200 µL and mixed well. Then, 100 µL from the first column was transferred to the second column with a final volume of 200 µL (two-fold dilution) and mixed well. The steps of two-fold serial dilution were repeated up to the tenth column with a final volume of 200 µL, producing concentration ranges of 0.01–25 µg/mL for the antibiotic solution and 1.56–3200 µg/mL for the extract solution. One hundred microliters from the tenth column was discarded with a final volume of 100 µL for each well. One hundred microliters of Leptospira culture was added into the first column until the tenth column with a final volume of 200 µL for each well. Positive (100 µL broth and 100 µL Leptospira) and negative control (200 µL broth) of 13 wells were included in the eleventh and twelfth columns, respectively. A growth control plate was prepared by replacing Leptospira cultures with broth. The plates were incubated at 30 °C and 40 rpm for seven days. The absorbance of the test plate was read by an automatic enzyme-linked immunosorbent assay (ELISA) tray reader at 420 nm. To eliminate interferences, the control absorbance value was subtracted from the total absorbance values, and the pre-incubation absorbance values were subtracted from the post-incubation values. Turbidity and an obvious white dot at the bottom of wells showed Leptospira growth, and this was confirmed by examining a loopful of each well's contents under a dark field microscope at 200× magnification, zooming in and out [15, 16]. Wells of MICs and the next two dilutions of increased concentrations were proceeded to MBC determination by inoculating a loopful of each well onto the agar EMJH medium and incubating at 30 °C for two to three weeks. Each experiment was triplicated to ensure reliability.

2.6 Synergy Testing by Checkerboard Assay

For MIC testing of the extract, 100 µL EMJH broth was added to the first column of a 96-well plate. One hundred microliters of extract was added to E1 well with a final volume of 200 µL. The extract was serially diluted by transferring 100 µL of the extract solution from E1 to the next well by two-fold. Then, 100 µL of Leptospira cultures was added to the first column of the 96-well plate (final volume of 200 µL per well). For MIC testing of the antibiotic, 100 µL EMJH broth was added to the first row of the 96-well plate. One hundred microliters of the antibiotic was added to A6 well with a final volume of 200 µL. The antibiotic was serially diluted by transferring 100 µL of the antibiotic solution from A6 to the next well by two-fold. Then, 100 µL Leptospira cultures were added to the first row of the 96-well plate (final volume of 200 µL per well). For synergy testing, the two-fold serial dilutions of 50 µL antibiotic and 50 µL extract were prepared and transferred into the second column until the twelfth column. Next, 100 µL of Leptospira culture was added into the respective columns (final volume of 200 µL per well) (Figure 6). Control plates were prepared with broth to replace Leptospira cultures. The plates were incubated at 30 °C and 40 rpm for seven days. The control absorbance value was subtracted from the total absorbance value determined by an ELISA reader, and the pre-incubation values were subtracted from the post-incubation values. Each of the synergy testing was duplicated. Turbidity and an obvious white dot at the bottom of wells showed Leptospira growth, and this was confirmed by examining a loopful of each

well's contents under a dark field microscope at 200×magnification, zooming in and out [15, 16]. Fractional inhibitory concentration index (FICI) values were calculated for the wells with the lowest concentration showing no visible growth using formulas 1 and 2, and interpreted according to criteria of the American Society of Microbiology and the British Society of Antimicrobial Chemotherapy as follows: synergy (FICI ≤ 0.5), additive (FICI > 0.5–1.0), indifferent (FICI > 1.0–4.0), and antagonistic (FICI > 4.0) [17, 18]:

$$\text{FICI of well} = \text{FIC of Antibiotic} + \text{FIC of extract} = \left(\frac{Concn\ of\ Antibiotic}{MIC\ of\ Antibiotic}\right) + \left(\frac{Concn\ of\ Extract}{MIC\ of\ Extract}\right) \quad (1)$$

$$FICI\ of\ test = \frac{\sum FICI\ of\ Wells}{Numberof\ well} \quad (2)$$

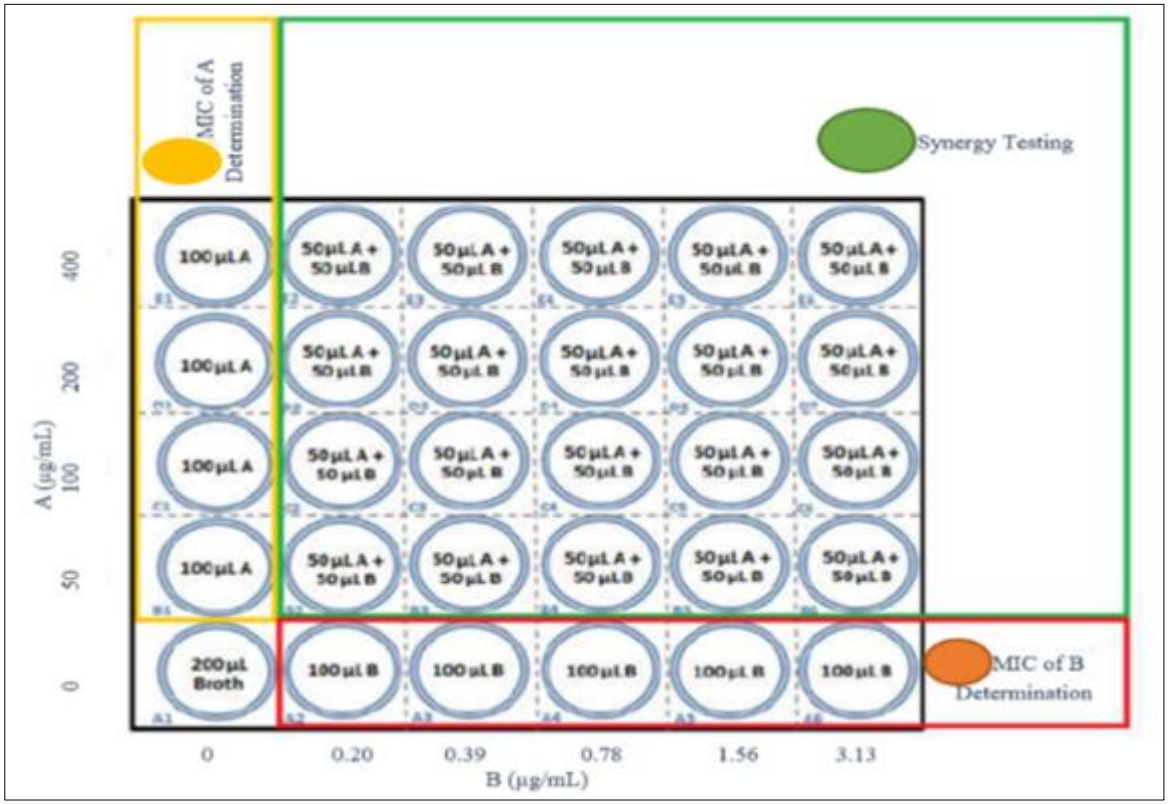

Figure 6 The arrangement of the synergy testing in a 96-well plate A=extract, B= Antibiotic (For five solvent extracts)

2.7 Scanning Electron Microscope Analysis

The combinations with the lowest FICI values were examined using scanning electron microscopy (SEM). *Leptospira* cultures (2 × 106 CFU/mL) were incubated at 30 °C and 40 rpm for seven days and treated with the MICs of the extracts and antibiotic solutions separately and combined for 18 h. A negative control (untreated *Leptospira*) sample was prepared. The samples were centrifuged (4560 rpm at 2000 g for 10 min) and the pellets were fixed using McDowell-Trumps fixative at 4^{o}c for two hours, centrifuged again, washed with 0.1 M PBS, and fixed using 1% osmium tetroxide at 4^{o}c for one hour. The pellets were then washed with 0.1 M PBS and dehydrated using 50%, 75%, 95%, and 100% acetone for ten minutes each. Then, 100% hexamethyldisilazane was added to the pellets for 10 min and air-dried overnight. Specimens were then mounted on sample stubs and coated with gold for examination with a high-resolution versatile SEM instrument (FEI, Brno, Czech Republic).

2.8 Statistical Analysis

The data were analysed using SPSS software version and presented as Mean±SD of the triplicates for antimicrobial susceptibility testing and duplicates for synergy testing.

3. Results

3.1 Antimicrobial Susceptibility Testing

The Minimum inhibitory concentration (MICs) and the minimum bactericidal concentration (MBCs) of the extracts and antibiotics are as shown in Tables 1-8 respectively. The crude solvent extracts from *Annona senegalensis* ranging from 100 µg/ml to 800 µg/ml showed significant anti-leptospiral activity. However strong activity was obtained from 400 µg/ml to 800 µg/ml. Benzathine Benzylpenicillin, Amoxicillin, Azithromycin, Ceftriaxone and Tetracycline

Benzathine Benzylpenicillin is used to treat a wide variety of bacterial infections. It may also be used to prevent certain bacterial infections (such as rheumatic fever). This medication is a long-acting penicillin antibiotic. It works by stopping the growth of bacteria.

Amoxicillin is used to treat a wide variety of bacterial infections. This medication is a penicillin-type antibiotic. It works by stopping the growth of bacteria.

Azithromycin is used to treat ma ny different types of infections caused by bacteria, such as respiratory infections, skin infections, ear infections, eye infections, and sexually transmitted diseases.

Ceftriaxone is a cephalosporin (SEF a low spor in) antibiotic that is used to treat many kinds of bacterial infections, including severe or life-threatening forms such as E. coli, pneumonia, or meningitis. Ceftriaxone is also used to prevent infection in people having certain types of surgery.

Tetracycline is an antibiotic that fights infection caused by bacteria. Tetracycline is used to treat many different bacterial infections of the skin, intestines, respiratory tract, urinary tract, genitals, lymph nodes, and other body systems.

Doxycycline is a tetracycline antibiotic that fights bacteria in the body. Doxycycline is used to treat many different bacterial infections, such as acne, urinary tract infections, intestinal infections, respiratory infections, eye infections, gonorrhoea, chlamydia, syphilis, and periodontitis.

Benzathine Benzylpenicillin showed the strongest anti-leptospiral activity, with MICs and MBCs ranging from 0.01 µg/mL to 0.88 µg/mL and 0.03 µg/mL to 4.15 µg/mL respectively, followed by ceftriaxone with both MICs and MBCs ranging from 0.06 to 0.77 µg/ml and Azithromycin, with MICs and MBCs ranging from 0.43 µg/mL to 4.12 µg/mL and 10.6 µg/mL to 27.13 µg/mL.

Table 1 and Table 2 Showing the MIC and MBC values (mean±SD) of *Annona senegalensis* solvents crude extracts on *Laptospira serovars* in triplicate. While Table 3 and Table 4 showing the MIC and MBC values (Mean±SD) of *Antibiotic* on *Laptospira serovars* in triplicate.

3.2 Synergy Testing

Table 1 The MIC values (Mean±SD) of *Annona senegalensis* solvents crude extracts (ASH, ASD, ASC, ASEt, ASM and ASEtol) on *Laptospira serovars javanica* in triplicate

Laptospira Interrogans serovars	**Minimum Inhibitory Concentration (MIC)**					
	Mean±SD (µg/mL)					
	ASH	**ASD**	**ASC**	**ASEt**	**ASM**	**ASEtol**
Javanica	400±0.0	400±0.0	400±0.0	800±0.0	**800±0.0**	800±0.0
Caniola	100±0.0	200±0.0	100±0.0	300±0.0	400±0.0	400±0.0
Bataviae	100±0.0	200±0.0	100±0.0	400±0.0	400±0.0	400±0.0
Australis	200±0.0	300±0.0	200±0.0	800±0.0	800±0.0	800±0.0

Tables 5-16 show the FICI values and antibacterial effects of extracts combined with antibiotics. *Annona senegalensis* [Hexane (ASH), Dichloromethane (ASD), Chloroform (ASC) Ethyl acetate (ASEt), Methanol (ASM) and Ethanol (ASEtol)]

combined with each of the antibiotic (Benzathine Benzylpenicillin, Amoxicillin, Azithromycin, Ciftraxone, Tetracycline and Doxycycline) exhibited indifferent effects against *Leptospira serovars*, while others exhibited indifferent or antagonistic activities

Table 2 The MBC values (Mean±SD) of *Annona senegalensis* solvents crude extracts (ASH, ASD, ASC, ASEt, ASM and ASEtol) on *Laptospira serovars javanica* in triplicate

Laptospira Interrogans serovars	**Minimum Bactericidal Concentration (MBC)**					
	Mean±SD (µg/mL) Laptospira serovars Javanica					
	ASH	ASD	ASC	ASEt	ASM	ASEtol
Javanica	600±0.0	600±0.0	800±0.0	800±0.0	**800±0.0**	800±0.0
Caniola	200±0.0	400±0.0	400±0.0	400±0.0	400±0.0	400±0.0
Bataviae	200±0.0	400±0.0	400±0.0	400±0.0	400±0.0	400±0.0
Australis	400±0.0	800±0.0	800±0.0	800±0.0	800±0.0	800±0.0

Table 3 The MIC values (Mean±SD) of *Antibiotic* on *Laptospira serovars* in triplicate

Laptospira interrogans serovars	**Minimum Inhibitory Concentration (MIC)**					
	Mean±SD (µg/mL)					
	Benzathine Benzylpenicillin	Amoxicillin	Azithromycin,	Ceftriaxone	Tetracycline.	Doxycycline
Javanica	**0.01±0.00**	0.03±0.00	1.12±0.00	0.02±0.00	0.03±0.00	0.86±0.00
Caniola	0.02±0.00	0.06±0.00	2.04±0.00	0.03±0.00	0.04±0.00	0.41±0.00
Bataviae	0.01±0.00	1.03±0.00	0.98±0.00	0.02±0.00	0.79±0.00	1.76±0.00
Australis	0.12±0.00	1.22±0.00	1.34±0.00	0.32±0.00	0.54±0.00	4.02±0.00

Table 4 The MBC values (Mean±SD) of Antibiotic on *Laptospira serovars* in triplicate

Laptospira serovars	**Minimum Bactericidal Concentration (MBC)**					
	Mean±SD (µg/mL)					
	Benzathine Benzylpenicillin	**Amoxicillin**	**Azithromycin,**	**Ceftriaxone**	**Tetracycline.**	**Doxycycline**
Javanica	**0.02±0.00**	0.03±0.00	0.01±0.00	0.41±0.00	0.03±0.00	13.45±0.00
Caniola	0.14±0.00	0.37±0.00	0.12±0.00	0.08±0.00	0.12±0.00	12.76±0.00
Bataviae	0.08±0.00	0.11±0.00	0.17±0.00	0.49±0.00	0.18±0.00	12.58±0.00
Australis	0.47±0.00	0.78±0.00	0.38±0.00	0.97±0.00	0.55±0.00	19.78±0.00

Table 5 FICL values of ASH, ASD and ASC in combination with Benzathine Benzylpenicillin (BBP) against *Leptospira sarovars*

Laptospira serovars	**Minimum Inhibitory Concentration (MIC) Synergy Testing**											
	Mean±SD (µg/mL) Combination of extracts; ASH, ASD, ASC and Benzathine Benzylpenicillin (BBP)											
	MIC ASH (µg/mL)	**MIC BBP (µg/mL)**	**FICL**	**ACT**	**MIC ASD (µg/mL)**	**MIC BBP (µg/mL)**	**FICL**	**ACT**	**MIC ASC (µg/mL)**	**MIC BBP (µg/mL)**	**FICL**	**ACT**
Javanica	200±0.00	0.03±0.00	3.00±0.00	IDF	200±0.00	0.02±0.00	3.04±0.00	IDF	200±0.00	0.03±0.00	1.46±0.00	IDF
Caniola	100±0.00	0.03±0.00	3.56±0.00	IDF	50±0.00	0.02±0.00	2.16±0.00	IDF	200±0.00	0.02±0.00	2.98±0.00	ATG
Bataviae	100±0.00	0.06±0.00	1.98±0.00	IDF	100±0.00	0.12±0.00	1.76±0.00	IDF	200±0.00	0.04±0.00	2.57±0.00	IDF
Australis	100±0.00	0.86±0.00	2.77±0.00	IDF	100±0.00	1.32±0.00	2.45±0.00	IDF	100±0.00	0.06±0.00	2.44±0.00	IDF

Activity (ACT) = Indifferent (IDF), Antagonistic (ATG)

Table 6 FICL values of ASH, ASD and ASC in combination with Benzathine Benzylpenicillin (BBP) against *Leptospira sarovars*

Laptospira serovars	**Minimum Inhibitory Concentration (MIC) Synergy Testing**											
	Mean±SD (µg/mL) Combination of extracts; ASEt, ASM, ASEtol and Benzathine Benzylpenicillin (BBP)											
	MIC ASEt (µg/mL)	**MIC BBP (µg/mL)**	**FICL**	**ACT**	**MIC ASM (µg/mL)**	**MIC BBP (µg/mL)**	**FICL**	**ACT**	**MIC ASEtol (µg/mL)**	**MIC BBP (µg/mL)**	**FICL**	**ACT**
Javanica	800±0.00	0.02±0.00	3.44±0.00	IDF	800±0.00	0.89±0.00	3.32±0.00	IDF	800±0.00	0.01±0.00	3.17±0.00	IDF
Caniola	400±0.00	0.01±0.00	3.11±0.00	IDF	400±0.00	0.01±0.00	3.16±0.00	IDF	400±0.00	0.01±0.00	3.12±0.00	IDF
Bataviae	400±0.00	0.03±0.00	5.00±0.00	ATG	200±0.00	0.29±0.00	4.78±0.00	ATG	400±0.00	0.48±0.00	4.89±0.00	ATG
Australis	400±0.00	0.03±0.00	3.49±0.00	IDF	400±0.00	0.01±0.00	3.66±0.00	IDF	800±0.00	0.75±0.00	3.57±0.00	IDF

Activity (ACT) = Indifferent (IDF), Antagonistic (ATG)

Table 7 FICL values of ASH, ASD and ASC in combination with Amoxicillin (AMX) against *Leptospira sarovars*

Laptospira serovars	**Minimum Inhibitory Concentration (MIC) Synergy Testing**											
	Mean±SD (µg/mL) Combination of extracts; ASH, ASD, ASC and Amoxicillin (AMX)											
	MIC ASH (µg/mL)	**MIC AMX (µg/mL)**	**FICL**	**ACT**	**MIC ASD (µg/mL)**	**MIC AMX (µg/mL)**	**FICL**	**ACT**	**MIC ASC (µg/mL)**	**MIC AMX (µg/mL)**	**FICL**	**ACT**
Javanica	800±0.00	0.79±0.00	1.60±0.00	IDF	800±0.00	2.05±0.00	2.11±0.00	IDF	800±0.00	0.01±0.00	3.33±0.00	IDF
Caniola	100±0.00	0.83±0.00	3.10±0.00	IDF	100±0.00	4.00±0.00	2.98±0.00	IDF	400±0.00	0.02±0.00	3.12±0.00	IDF
Bataviae	100±0.00	1.66±0.00	2.24±0.00	IDF	200±0.00	3.78±0.00	4.02±0.00	ATG	400±0.00	0.06±0.00	4.98±0.00	ATG
Australis	100±0.00	1.66±0.00	2.26±0.00	IDF	100±0.00	3.56±0.00	4.12±0.00	ATG	400±0.00	0.07±0.00	3.55±0.00	IDF

Activity (ACT) = Indifferent (IDF), Antagonistic (ATG)

Table 8 FICL values of ASH, ASD and ASC in combination with Amoxicillin (AMX) against *Leptospira sarovars*

Laptospira serovars	**Minimum Inhibitory Concentration (MIC) Synergy Testing**											
	Mean±SD (µg/mL) Combination of extracts; ASEt, ASM, ASEtol and Amoxicillin (AMX)											
	MIC ASEt (µg/mL)	**MIC AMX (µg/mL)**	**FICL**	**ACT**	**MIC ASM (µg/mL)**	**MIC AMX (µg/mL)**	**FICL**	**ACT**	**MIC ASEtol (µg/mL)**	**MIC AMX (µg/mL)**	**FICL**	**ACT**
Javanica	800±0.00	0.01±0.00	2.77±0.00	IDF	800±0.00	0.01±0.00	3.33±0.00	IDF	800±0.00	0.02±0.00	1.65±0.00	IDF
Caniola	400±0.00	0.01±0.00	3.63±0.00	IDF	400±0.00	0.01±0.00	3.12±0.00	IDF	400±0.00	0.01±0.00	0.11±0.00	IDF
Bataviae	400±0.00	0.05±0.00	1.49±0.00	IDF	400±0.00	0.01±0.00	5.00±0.00	ATG	400±0.00	1.46±0.00	0.02±0.00	IDF
Australis	800±0.00	0.77±0.00	1.58±0.00	IDF	400±0.00	0.05±0.00	2.98±0.00	IDF	800±0.00	1.57±0.00	1.56±0.00	ATG

Activity (ACT) = Indifferent (IDF), Antagonistic (ATG)

Table 9 FICL values of ASH, ASD and ASC in combination with Azithromycin (AZT) against *Leptospira sarovars*

Laptospira serovars	Minimum Inhibitory Concentration (MIC) Synergy Testing											
	Mean±SD (µg/mL) Combination of extracts; ASEt, ASM, ASEtol and Amoxicillin (AMX)											
	MIC ASH (µg/mL)	**MIC AZT (µg/mL)**	**FICL**	**ACT**	**MIC ASD (µg/mL)**	**MIC AZT (µg/mL)**	**FICL**	**ACT**	**MIC ASC (µg/mL)**	**MIC AZT (µg/mL)**	**FICL**	**ACT**
Javanica	400±0.00	0.20±0.00	2.59±0.00	IDF	400±0.00	0.20±0.00	2.89±0.00	IDF	800±0.00	**0.01±0.00**	3.22±0.00	IDF
Caniola	400±0.00	0.05±0.00	1.98±0.00	IDF	400±0.00	0.02±0.00	2.66±0.00	IDF	800±0.00	**0.01±0.00**	2.99±0.00	IDF
Bataviae	200±0.00	0.01±0.00	2.46±0.00	IDF	100±0.00	0.20±0.00	2.76±0.00	IDF	400±0.00	**0.05±0.00**	5.00±0.00	ATG
Australis	400±0.00	1.64±0.00	2.60±0.00	IDF	400±0.00	1.67±0.00	2.81±0.00	IDF	800±0.00	**0.86±0.00**	3.76±0.00	IDF

Activity (ACT) = Indifferent (IDF), Antagonistic (ATG)

Table 10 FICL values of ASH, ASD and ASC in combination with Azithromycin (AZT) against *Leptospira sarovars*

Laptospira serovars	Minimum Inhibitory Concentration (MIC) Synergy Testing											
	Mean±SD (µg/mL) Combination of extracts; ASEt, ASM, ASEtol and Azithromycin (AZT)											
	MIC ASH (µg/mL)	**MIC AZT (µg/mL)**	**FICL**	**ATG**	**MIC ASD (µg/mL)**	**MIC AZT (µg/mL)**	**FICL**	**ACT**	**MIC ASC (µg/mL)**	**MIC AZT (µg/mL)**	**FICL**	**ATG**
Javanica	400±0.00	0.11±0.00	2.45±0.00	IDF	400±0.00	0.15±0.00	3.24±0.00	IDF	400±0.00	3.19±0.00	3.11±0.00	IDF
Caniola	100±0.00	0.01±0.00	2.44±0.00	IDF	400±0.00	0.01±0.00	2.33±0.00	IDF	100±0.00	3.32±0.00	2.36±0.00	IDF
Bataviae	100±0.00	0.05±0.00	2.46±0.00	IDF	200±0.00	0.02±0.00	3.56±0.00	IDF	100±0.00	2.77±0.00	3.41±0.00	IDF
Australis	400±0.00	0.10±0.00	2.33±0.00	IDF	400±0.00	1.63±0.00	2.86±0.00	IDF	400±0.00	3.44±0.00	2.77±0.00	IDF

Activity (ACT) = Indifferent (IDF), Antagonistic (ATG)

Table 11 FICL values of ASH, ASD and ASC in combination with Ceftriaxone (CFT) against *Leptospira sarovars*

Laptospira serovars	Minimum Inhibitory Concentration (MIC) Synergy Testing											
	Mean±SD (µg/mL) Combination of extracts; ASH, ASD, ASC and Ceftriaxone (CFT)											
	MIC ASH (µg/mL)	MIC CFT (µg/mL)	FICL	ATG	MIC ASD (µg/mL)	MIC CFT (µg/mL)	FICL	ACT	MIC ASC (µg/mL)	MIC CFT (µg/mL)	FICL	ACT
Javanica	400±0.00	0.12±0.00	2.44±0.00	IDF	800±0.00	0.38±0.00	2.78±0.00	IDF	800±0.00	0.43±0.00	2.64±0.00	IDF
Caniola	100±0.00	0.06±0.00	2.33±0.00	IDF	400±0.00	0.38±0.00	1.56±0.00	IDF	200±0.00	0.39±0.00	3.24±0.00	IDF
Bataviae	100±0.00	0.18±0.00	2.39±0.00	IDF	400±0.00	1.44±0.00	0.86±0.00	IDF	200±0.00	0.39±0.00	2.56±0.00	IDF
Australis	200±0.00	1.64±0.00	1.58±0.00	IDF	800±0.00	0..89±0.00	2.63±0.00	IDF	400±0.00	0.88±0.00	3.42±0.00	IDF

Activity (ACT) = Indifferent (IDF), Antagonistic (ATG)

Table 12 FICL values of ASH, ASD and ASC in combination with Ceftriaxone (CFT) against *Leptospira sarovars*

Laptospira serovars	Minimum Inhibitory Concentration (MIC) Synergy Testing											
	Mean±SD (µg/mL) Combination of extracts; ASEt, ASM, ASEtol and Ceftriaxone (CFT)											
	MIC ASH (µg/mL)	MIC CFT (µg/mL)	FICL	ACT	MIC ASD (µg/mL)	MIC CFT (µg/mL)	FICL	ACT	MIC ASC (µg/mL)	MIC CFT (µg/mL)	FICL	ACT
Javanica	800±0.00	1.56±0.00	1.43±0.00	IDF	800±0.00	0.39±0.00	2.82±0.00	IDF	400±0.00	0.48±0.00	0.79±0.00	IDF
Caniola	400±0.00	0.02±0.00	1.56±0.00	IDF	400±0.00	0.38±0.00	4.32±0.00	ATG	200±0.00	0.39±0.00	1.67±0.00	IDF
Bataviae	400±0.00	0.01±0.00	0.01±0.00	IDF	400±0.00	1.23±0.00	2.63±0.00	IDF	200±0.00	0.39±0.00	1.56±0.00	IDF
Australis	800±0.00	1.56±0.00	1.62±0.00	IDF	400±0.00	0.87±0.00	3.89±0.00	IDF	800±0.00	0.24±0.00	0.42±0.00	IDF

Activity (ACT) = Indifferent (IDF), Antagonistic (ATG)

Table 13 FICL values of ASH, ASD and ASC in combination with Tetracycline (TTC) against *Leptospira sarovars*

Laptospira serovars	Minimum Inhibitory Concentration (MIC) Synergy Testing											
	Mean±SD (µg/mL) Combination of extracts; ASH, ASD, ASC and Tetracycline (TTC)											
	MIC ASH (µg/mL)	MIC TTC (µg/mL)	FICL	ACT	MIC ASD (µg/mL)	MIC TTC (µg/mL)	FICL	ACT	MIC ASC (µg/mL)	MIC TTC (µg/mL)	FICL	ACT
Javanica	800±0.00	0.01±0.00	3.42±0.00	IDF	400±0.00	1.46±0.00	1.58±0.00	IDF	400±0.00	1.63±0.00	2.56±0.00	IDF
Caniola	400±0.00	0.01±0.00	2.78±0.00	IDF	200±0.00	0.01±0.00	0.41±0.00	IDF	50±0.00	0.73±0.00	3.44±0.00	IDF
Bataviae	400±0.00	0.05±0.00	4.87±0.00	ATG	200±0.00	0.39±0.00	0.39±0.00	IDF	100±0.00	1.57±0.00	2.56±0.00	IDF
Australis	400±0.00	0.89±0.00	3.83±0.00	IDF	400±0.00	2.14±0.00	0.89±0.00	IDF	400±0.00	0.39±0.00	0.79±0.00	IDF

Activity (ACT) = Indifferent (IDF), Antagonistic (ATG)

Table 14 FICL values of ASH, ASD and ASC in combination with Tetracycline (TTC) against *Leptospira sarovars*

Laptospira serovars	Minimum Inhibitory Concentration (MIC) Synergy Testing											
	Mean±SD (µg/mL) Combination of extracts; ASEt, ASM, ASEtol and Tetracycline (TTC)											
	MIC ASEt (µg/mL)	MIC TTC (µg/mL)	FICL	ACT	MIC ASM (µg/mL)	MIC TTC (µg/mL)	FICL	ACT	MIC ASEtol (µg/mL)	MIC TTC (µg/mL)	FICL	ACT
Javanica	800±0.00	0.01±0.00	3.45±0.00	IDF	200±0.00	0.02±0.00	3.43±0.00	IDF	200±0.00	0.02±0.00	0.89±0.00	IDF
Caniola	400±0.00	0.01±0.00	2.76±0.00	IDF	50±0.00	0.02±0.00	2.89±0.00	IDF	400±0.00	2.98±0.00	2.89±0.00	IDF
Bataviae	400±0.00	0.05±0.00	4.99±0.00	ATG	100±0.00	0.05±0.00	4.98±0.00	ATG	400±0.00	2.98±0.00	0.05±0.00	IDF
Australis	800±0.00	0.89±0.00	3.23±0.00	IDF	100±0.00	0.79±0.00	3.56±0.00	IDF	100±0.00	0.05±0.00	3.12±0.00	IDF

Activity (ACT) = Indifferent (IDF), Antagonistic (ATG)

Table 15 FICL values of ASH, ASD and ASC in combination with Doxycycline (DXC) against *Leptospira sarovars*

Laptospira serovars	Minimum Inhibitory Concentration (MIC) Synergy Testing											
	Mean±SD (µg/mL) Combination of extracts; ASH, ASD, ASC and Doxycycline (DXC)											
	MIC ASH (µg/mL)	MIC DXC (µg/mL)	FICL	ACT	MIC ASD (µg/mL)	MIC DXC (µg/mL)	FICL	ACT	MIC ASC (µg/mL)	MIC DXC (µg/mL)	FICL	ACT
Javanica	800±0.00	0.01±0.00	3.12±0.00	IDF	400±0.00	1.12±0.00	1.28±0.00	IDF	400±0.00	1.54±0.00	2.36±0.00	IDF
Caniola	400±0.00	0.05±0.00	2.34±0.00	IDF	200±0.00	0.01±0.00	0.31±0.00	IDF	50±0.00	0.78±0.00	3.14±0.00	IDF
Bataviae	400±0.00	0.12±0.00	3.87±0.00	IDF	200±0.00	0.34±0.00	0.29±0.00	IDF	100±0.00	1.24±0.00	2.46±0.00	IDF
Australis	400±0.00	0.76±0.00	2.83±0.00	IDF	400±0.00	2.10±0.00	0.79±0.00	IDF	400±0.00	0.78±0.00	0.89±0.00	IDF

Activity (ACT) = Indifferent (IDF), Antagonistic (ATG)

Table 16 FICL values of ASH, ASD and ASC in combination with Doxycycline (DXC) against *Leptospira sarovars*

Laptospira serovars	Minimum Inhibitory Concentration (MIC) Synergy Testing											
	Mean±SD (µg/mL) Combination of extracts; ASEt, ASM, ASEtol and Doxycycline (DXC)											
	MIC ASH (µg/mL)	MIC DXC (µg/mL)	FICL	ACT	MIC ASD (µg/mL)	MIC DXC (µg/mL)	FICL	ACT	MIC ASC (µg/mL)	MIC DXC (µg/mL)	FICL	ACT
Javanica	800±0.00	0.01±0.00	3.45±0.00	IDF	200±0.00	0.02±0.00	3.43±0.00	IDF	200±0.00	0.02±0.00	0.84±0.00	IDF
Caniola	100±0.00	0.07±0.00	0.76±0.00	IDF	100±0.00	0.23±0.00	1.89±0.00	IDF	400±0.00	2.56±0.00	2.67±0.00	IDF
Bataviae	100±0.00	0.25±0.00	2.45±0.00	IDF	100±0.00	0.12±0.00	2.98±0.00	IDF	400±0.00	2.56±0.00	0.05±0.00	IDF
Australis	800±0.00	0.89±0.00	3.23±0.00	IDF	400±0.00	0.05±0.00	3.56±0.00	IDF	100±0.00	0.05±0.00	3.10±0.00	IDF

Activity (ACT) = Indifferent (IDF), Antagonistic (ATG)

3.3 SEM Analysis

Figures 7-11 shows the SEM micrographs of structural difference between the treated and untreated Leptospira with Benzathine Benzylpenicillin (BBP) and *Annona senegalensis* methanol crude extract (ASM), against *Leptospira sarovars*. The SEM micrographs of the control Leptospira cells showed normal spirochete morphology with helical-shaped and hooked-end structures (Figure 7). Leptospira treated with the MIC of Benzathine Benzylpenicillin (BBP) 0.89±0.00 µg/mL of FICI 3.32±0.00 of indifferent activity (IDF)

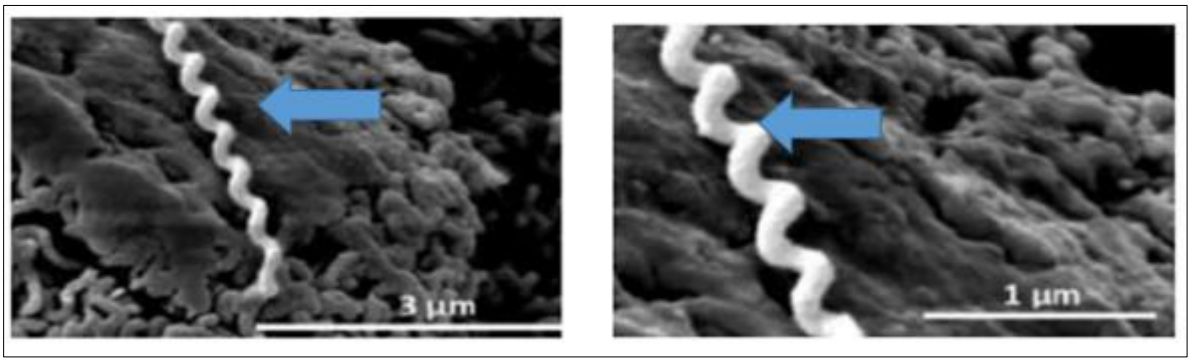

Figure 7 SEM micrographs of normal L interrogans serovar *Javanica* at 30,000Mag (i) and 60,000 Mag (ii) with a standard helical shape

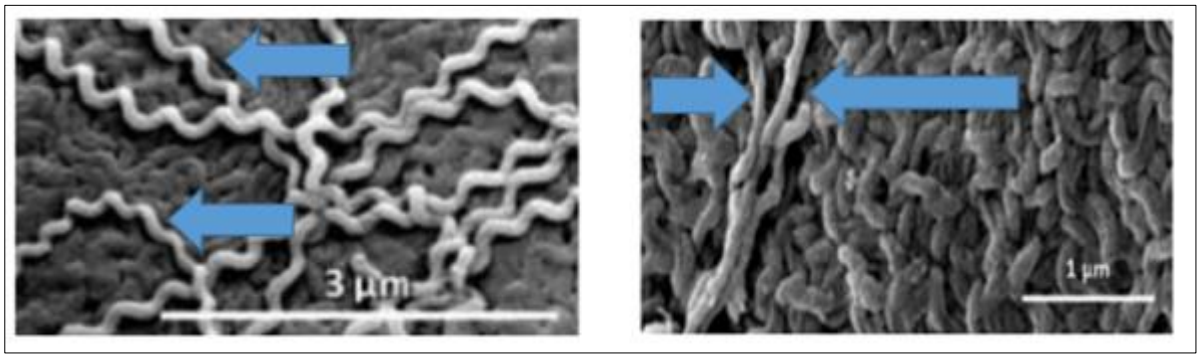

Figure 8 SEM micrograph of L interrogans serovar *Javanica* treated with the MIC of Benzathine Benzylpenicillin (BBP less coiling and irregular structure observed

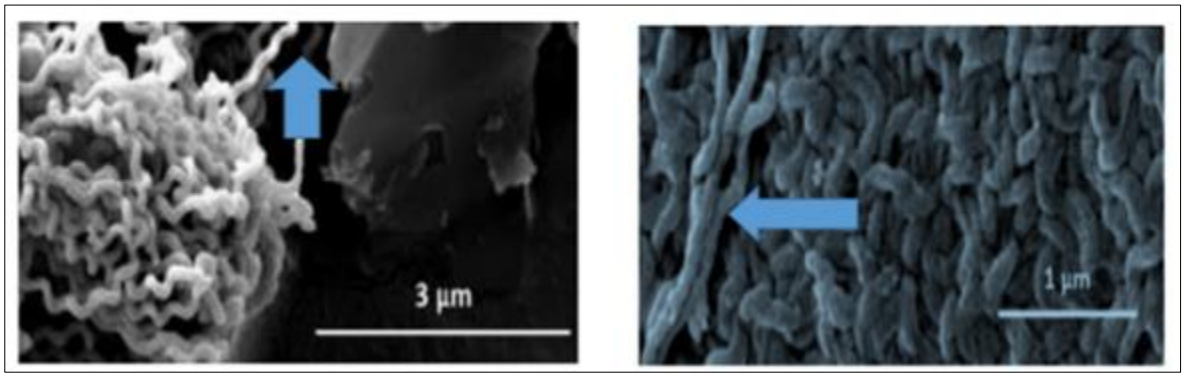

Figure 9 SEM micrograph of L interrogans serovar *Javanica* treated with treated with the MIC of ASM at 30,000 (i) and 60,000 (ii) magnification, less coiling was observed and irregular structure of the Leptospira cells were obtained

Exhibited thinning and less coiling and irregular surface (Figure 8) while at the same time those treated with Benzathine Benzylpenicillin (BBP) and *Annona senegalensis* ethanol crude extract (ASEtol), against *Leptospira sarovars* at 800 µg/mL exhibited less coiling and irregular surface Figure 9 and those treated with Benzathine Benzylpenicillin (BBP) and *Annona senegalensis* ethanol crude extract (ASEtol), against *Leptospira sarovars* exhibited severe damage (Figure 10) with a thinning structure which is short, less and distorted coiling. There is also an appearance of irregular and blebby surface.

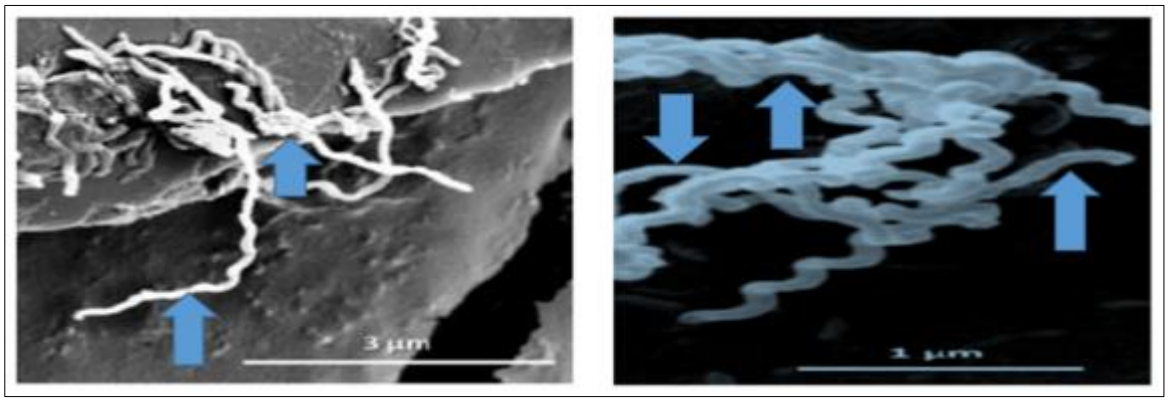

Figure 10 SEM micrograph of L interrogans serovar *Javanica* treated with treated with Benzathine Benzylpenicillin (BBP) and *Annona senegalensis* ethanol crude extract (ASM), show less coiling and distorted surface, thinning and shortened cell, irregular and blabbing structure was observed

4. Discussion

The result from this study suggest that extracts from this plant *Annona senegalensis* at different solvent extracts (ASH, ASD, ASC, ASEt, and ASM) exhibited a significant anti-leptospiral effects, to some extent the MIC ranges of the solvent extracts coincided with the MBC ranges suggesting that the same concentration of *Annona senegalensis* simultaneously inhibits and completely kills the Leptospira. In this study it was found that the extract from methanol crude extract (ASM) and the Ethanol crude extract (ASEtol) were more effective than the Hexane (ASH), Dichloromethane (ASD), Chloroform (ASC) and Ethyle acetate (ASEt) against the bacteria Leptospiral. Other studies have also reported that ASM and ASEtol are more effective and showed more potential on bacteria as well as fungi like *Bacillus subtilis, Pseudomonas aerginosa, Escherichia coli, Salmonella typhi*, *Staphylococcus aureus* and *Candida albicans* and *Aspergillus niger, Aspergillus flavus, Fusarium oxysporum, and Rhizopus stolonifer* then the ASH, ASD, and ASC. [19, 20, 21].

The preliminary research of our study found differences in the anti leptospiral effects between the different solvent extracts. This suggest that the solvent extraction process and the choice of solvents play a great role in the chemical components that it best inhibit the growth of the bacteria Laptospiral. With the presence of this chemical constituent at various solvents extract based on polarity rate, these chemical constituents such as steroids exert antibacterial effects, mainly by disrupting the formation and structure of bacteria cells, causing them to increase their fluidity. These changes lead to an uncontrolled efflux of metabolites, ions, and membrane proteins, causing leakage of cell contents, resulting in cell damage, lysis, and eventually cell death. Flavonoids, saponins, tannins, and anthraquinones disrupt the microbial cell wall by increasing its permeability, leading to cytoplasm leakage [24, 25, and 26]. Terpenoids, alkaloids, tannins, and anthraquinones inhibit biofilm formation, thus reducing resistance to antibacterial agents and environmental conditions. Terpenoids, alkaloids, saponins, and tannins inhibit microbial growth by disrupting microbial cell physiology and metabolism, including the inhibition of protein, nucleic acid, and adenosine triphosphate synthesis [27-34]. This actions might be applied to *Leptospira* as well. The effects observed in this study might be exerted not only by their major components separately, but also by their synergism reaction.

Our study found that the tested antibiotics were more effective in inhibiting Leptospira growth than *Annona senegalensis* extracts. This can be explained by the fact that they are in pure form, whereas crude plant extracts contain substances that may not be particularly effective in inhibiting Leptospira growth or killing the bacteria. Leptospira serovars were more susceptible to beta-lactam antibiotics, primarily Benzathine Benzylpenicillin and to a lesser degree Azithromycin, Ceftriaxone and less susceptible to Amoxicillin, Tetracycline and Doxycycline. Similar findings have been reported by previous studies [35-37]. In our study, Benzathine Benzylpenicillin was most effective against L. interrogans serovar Bataviae, followed by serovars Australis, Canicola, and Javanica. Thus, from the high MBC ranges of

Amoxicillin, Tetracycline and Doxycycline observed in this study suggest that higher concentration is required to kill the Leptospira completely.

In this study, Benzathine Benzylpenicillin, Azithromycin and ceftriaxone was most effective against L. interrogans serovar Javanica, Bataviae, followed by serovars Australis, and Canicola. The MICs and MBCs against L. interrogans serovars suggesting that it inhibits and kills the Leptospira at the different concentrations. Benzathine Benzylpenicillin, Azithromycin, and ceftriaxone was found to be effective against all Leptospira spp. and most effective against L. interrogans serovar Australis, followed by serovars Bataviae, Canicola, and Javanica. In line with our findings, previous studies have reported that L. interrogans serovar Bataviae is more susceptible to penicillin G, as well as to doxycycline, than L. interrogans serovar Canicola [37-39].

Our study found indifferent inhibitory effects of combinations of antibiotics and extracts against Leptospira serovars, except for the antagonistic effect of *Annona senegalensis* ASM, ASEt and ASEtol combined with Benzathine Benzylpenicillin, Azithromycin and ceftriaxone on L. interrogans serovar. The lowest FICI value was observed among the ASH, ASD and ASC combined with the selected antibiotic against L. interrogans serovar, this was found to be close to additive effect values (>0.5–1.0), suggesting that *Annona senegalensis* extracts could have stronger effects when in a purified form. There are limited data on the synergistic effects of plant extracts against Leptospira spp. and no data on *Annona senegalensis* extracts of different solvents.

In this study, SEM of the methanol (ASM) revealed that combination with Benzathine Benzylpenicillin, caused damage to L. interrogans serovar Javanica. Cell bulging or blebbing is a known effect of Benzathine Benzylpenicillin, distortions of the spirochaete, Bacterial blebbing, which is one of the factors leading to cell lysis, is caused by physiological stress and turgor pressure, metabolite depletion, cell ageing, exposure to antibiotics, and pH changes that disturb the cell envelope [40,41]. In living cells, cell blebs transport microbial virulence factors and are involved in cell communication and division [40]. The mechanism of action of Benzathine Benzylpenicillin against L. interrogans serovar javanica is associated with thinning, shortening, bleb formation, and reduced coiling. To our knowledge, this is the first study to investigate the effects of *Annona senegalensis* extracts on bacterial morphology. The observed distorted Leptospira surface morphology could be due to the chemical constituents of *Annona senegalensis* extracts that mainly exert effects on the cellular membranes, which can be further analysed in the future research. Considering the synergy testing and SEM results, *Annona senegalensis* combined with antibiotic cause's severe damage to Leptospira cells but with no enhancement on its inhibiting and killing abilities on Leptospira.

5. Conclusion

Annona senegalensis showed promising anti-leptospiral properties, with ASEt, ASM and ASEtol yielding better results than ASH, ASD and ASC. All tested Leptospira serovars were more susceptible to most of the selected antibiotic. Synergy testing revealed moderate effects, indicating a potential effects against the disease. Thus, *Annona senegalensis* combined with the selected antibiotic most especially Benzathine Benzylpenicillin, Azithromycin and ceftriaxone caused significant damage to Leptospira cell morphology, suggesting the potential of *Annona senegalensis* as an anti-leptospiral agent. Further evaluation of *Annona senegalensis* in animal models study is needed, especially in Gashaka Gumti National Park animals as they have potential anti-leptospiral property, as well as to investigate the whole effects and mechanisms on the subjects including its toxicity.

Compliance with ethical standards

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Disclosure of conflict of interest

There is no competing interest.

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